



Cunha, A. F., Felipe, I. S. A., Ferreira-Junior, N. C., Resstel, L. B. M., Guimarães, D. A. M., Beijamini, V., Paton, J. F. R., & Sampaio, K. N. (2018). Neuroreflex control of cardiovascular function is impaired after acute poisoning with chlorpyrifos, an organophosphorus insecticide: Possible short and long term clinical implications. *Toxicology*, 398-399, 13-22. <https://doi.org/10.1016/j.tox.2018.02.005>

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[10.1016/j.tox.2018.02.005](https://doi.org/10.1016/j.tox.2018.02.005)

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**Neuroreflex control of cardiovascular function is impaired after
acute poisoning with chlorpyrifos, an organophosphorus
insecticide: Possible short and long term clinical implications.**

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Abstract

Although it is well-established that severe poisoning by organophosphorus (OP) compounds strongly affects the cardiorespiratory system, the effects of sub-lethal exposure to these compounds on the neural control of cardiovascular function are poorly explored. The aim of this study was to evaluate the effects of acute sub-lethal exposure to chlorpyrifos (CPF), a commonly used OP insecticide, on three basic reflex mechanisms involved in blood pressure regulation, the peripheral chemoreflex, the baroreflex and the Bezold-Jarisch reflex. Adult male Wistar rats were injected intraperitoneally with a single dose of CPF (30 mg/Kg) or saline (0.9%). 24 hours after injections, cardiovascular reflexes were tested in awake rats. Potassium cyanide (KCN) and phenylbiguanide (PBG) were intravenously injected to activate the chemoreflex and the Bezold-Jarisch reflex, respectively. The baroreflex was activated by phenylephrine and sodium nitroprusside infusions. Blood samples were taken for measurements of butyrylcholinesterase (BChE) activity while acetylcholinesterase (AChE) activity was measured in brainstem samples. Animals treated with CPF presented signs of intoxication such as ataxia, tremor, lacrimation, salivation, tetany, urination and defecation. The hypertensive and the bradycardic responses of the chemoreflex as well as the hypotensive and bradycardic responses of the Bezold-Jarisch reflex were all attenuated in CPF treated animals ($P < 0.05$). Concerning the baroreflex responses, CPF treatment reduced the bradycardia plateau, the range and the gain of the reflex ($P < 0.05$). Plasma BChE and brainstem AChE were both reduced significantly after CPF treatment ($P < 0.05$). Our results showed that acute sub-lethal exposure to CPF impairs the cardiovascular responses of homeostatic and defensive cardiovascular reflex. These effects are associated with a marked inhibition of plasma BChE and brainstem AChE.

Keywords: Chlorpyrifos; Baroreflex; Bezold-Jarisch reflex; Peripheral Chemoreflex; Butyrylcholinesterase; Acetylcholinesterase

Introduction

It is well established that severe poisoning by organophosphorus (OP) compounds produce marked pathological changes in the autonomic, somatic and central nervous systems (Tafari & Roberts 1987; Marrs 1993; Clark 2006; King & Aaron 2015). Chlorpyrifos (CPF), a common and highly used broad spectrum OP insecticide, exerts its predominant toxicological effect by inhibition of acetylcholinesterase (AChE), the enzyme that breaks down acetylcholine (Risher & Navarro 1998; Marty et al. 2012; Reiss et al. 2012). Similar to other OP compounds, inhibition of AChE leads to overstimulation of cholinergic nicotinic and muscarinic receptors giving rise to a wide range of potentially lethal side effects. In particular, interference with cardiorespiratory homeostasis may cause symptoms of, for example: hypotension, hypertension, bradycardia, tachycardia, ventricular arrhythmias, ECG changes, bronchoconstriction, increased bronchial secretions, respiratory paralysis, cyanosis and cardiac arrest (Marrs 1993; King & Aaron 2015). However, despite these prominent clinical features of OP poisoning, very few studies have elucidated the effects on reflex control mechanisms involved in regulating the cardiovascular system.

The maintenance of adequate tissue perfusion is achieved by an integrative action of short- and long term control mechanisms. Neural reflexes exert a moment to moment control of cardiovascular function to correct and maintain blood flow and oxygen supply to tissues (Spyer 1990; Guyenet 2006; Thomas 2011; Salman 2016). The baroreflex is one of the most important and powerful reflexes for regulating arterial pressure; this is performed by mechanoreceptors located within the aortic arch and the carotid sinuses, which mediate reflex responses through the brainstem and the parasympathetic and sympathetic outflow to the heart and vessels. When arterial pressure is increased, the consequent baroreceptor discharge promotes inhibition of the sympathetic drive to heart and vessels and activation of the parasympathetic outflow to the heart, decreasing vascular resistance, heart rate and cardiac output in order to bring pressure levels back to normal (Spyer 1990; Guyenet 2006; Thomas 2011; Salman 2016).

Despite the marked role played by the arterial baroreflex, cardiopulmonary receptors located within the heart, aorta and lungs are also involved in neural reflex control of the circulation (Zanchetti and Mancia, 1991). These receptors respond to either chemical or mechanical stimuli; activation of the former mediates the Bezold-Jarisch reflex (Aviado and Guevara Aviado, 2001). This reflex is activated by toxic and irritant agents promoting a powerful vagal mediated bradycardia due to parasympathetic activation and a hypotensive response due to withdrawal of sympathetic vasoconstriction tone and a decrease in cardiac output (Campagna and Carter, 2003; Salman, 2016; Sapru, 2002).

Changes in pO_2 , pCO_2 and pH can also trigger cardiorespiratory reflex responses by activation of peripheral and central chemoreceptors (Kara et al., 2003). The peripheral chemoreceptors are located in the carotid and aortic bodies and are mainly sensitive to hypoxic states (Prabhakar & Peng 2004; Nurse 2010). The cardiorespiratory responses are again mediated by an intricate reflex network that passes via the brainstem producing a dramatic increase in sympathetic outflow to vessels and in vagal discharge to the heart combined with a hyperventilatory response in order to restore oxygen levels (Machado, 2001; Barros et al., 2002; Sapru, 2002; Spyer & Gourine, 2009).

Malfunction of the integrative cardiorespiratory function of these reflexes is associated with the development and maintenance of pathological states such as hypertension, hypotension, heart failure, dyspnoea and apnoea (Zanchetti & Mancia, 1991; Vasquez et al. 1997; Guyenet 2006; Simms et al. 2007; Molkov et al. 2014; Fernandez et al. 2015). Previous studies from our laboratory showed that acute exposure to an OP compound, methamidophos, impaired the cardiovascular responses of two neural reflexes, the peripheral chemoreflex and the Bezold-Jarisch reflex (Maretto et al., 2012). However, the effects of OP exposure on the baroreflex regulation remained unexplored. Despite the common cholinergic interference shared by all OP compounds, there is evidence that cardiac, enzymatic and central interaction of these agents may vary according to the specific compound type (Howard and Pope, 2002; Kwong, 2002; Mirajkar and

Pope, 2008; Pope, 1999; Slotkin et al., 2006; Storm et al., 2000). Therefore, we decided to investigate the effects of an acute sub-lethal exposure to CPF, another OP insecticide, on the baroreflex, chemoreflex and the Bezold-Jarisch reflex. Since the main CNS nuclei involved in mediating these reflexes are located within the brain stem, we determined the AChE activity in this specific brain region as well as the plasma BChE; this allowed us to associate any changes in cardiovascular reflex function with the change in cholinergic transmission.

Methods

Animals

Adult male Wistar rats weighting between 350-400g were used. The animals were provided by the animal facilities of the Health Sciences Center of the Federal University of Espírito Santo. Rats were housed in groups of 5/cage at a temperature ($20-24 \pm 1^{\circ}\text{C}$) and humidity controlled room with a 12 h light/dark cycle (lights on at 6:30 a.m.). Standard chow and tap water were available *ad libitum*. All experiments were approved by the University Committee for the Ethical Use of Animals in Scientific Research (CEUA-UFES; approval numbers 013/2013 and 024/2013).

Catheterization Procedure and Pressure Recordings

The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p., Cristália Produtos Químicos Farmacêuticos Ltda, SP-Brazil) and a catheter (PE-10 connected to PE-20) was inserted into the abdominal aorta, through the femoral artery, to allow arterial pressure recordings and blood sampling. Another catheter was inserted into the femoral vein for administration of drugs to activate cardiovascular reflexes. Both catheters were tunneled subcutaneously and exteriorized through the back of the neck. Rats were allowed to recover overnight between surgical procedures and arterial pressure recordings. The pressure recordings were performed in awake rats using a pressure transducer interfaced to a computer recording system (Powerlab, ADInstruments, New Zealand). Pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) were recorded. The MAP and HR were derived from the pulsatile pressure and were analyzed off-line using a digital filter of 0.2Hz.

Chlorpyrifos (CPF) Treatment

CPF (Commercial formulation, Lorsban 480 BR, 48% w/v, Dow Agrosiences, Brazil) was diluted in saline (NaCl, 0.9% w/v, Vetec Química Fina Ltda, RJ-Brazil) for intraperitoneal injections (i.p.) Inert ingredients were also present in the composition of the commercial formulation and dilution in saline was promptly achieved. Control groups received injections of vehicle (saline, i.p.). Either CPF or

saline was given in a volume of 1 ml/kg. The dose of CPF (30 mg/kg) was chosen for treatment among five other doses tested (25, 30, 35, 40, 50, 60 mg/kg; data not shown) as it produced robust cholinergic signs without inducing lethality. For each dose tested, 10-15 animals were injected and the number of deaths counted within 24 hours. The CPF solution was freshly prepared and always used on the same day for each rat.

Acute toxicity

A pilot trial was conducted with 10 saline and 10 chlorpyrifos treated rats for evaluation of acute toxicity signs. Animals were observed for a period of up to three hours after treatment for signs of, for example: ataxia, tremors, lacrimation, salivation, tetany, urination and defecation. The presence or absence of these signs were recorded and the data expressed as percentage of animals that presented each specific behavior against the time since administration of saline or CPF (30 mg/kg). Acute toxicity signs were also monitored in all animals tested in the cardiovascular reflexes 24 hours after 30 mg/kg CPF dosing (N=30/treatment; see Experimental Protocol for description). The data from these animals were included with that obtained from those in the pilot trial, so a total of 40 rats/treatment were utilized.

Blood Samples

Blood samples were collected from the femoral artery catheter (0.1ml/collection) before and 24 hours after CPF treatment. Samples were collected in heparinized plastic microtubes and plasma extracted after centrifugation at 1792 G for 10 minutes at 4°C (SL-5AM - Spinlab Scientific, South Korea). Samples were used to measure the butyrylcholinesterase (BChE) activity, a recognized parameter to monitor OP exposure (Eddleston et al., 2008, 2002). Plasma samples were collected from CPF and vehicle treated rats and stored at -20°C until the day of the assay.

Brainstem Samples

Rats were killed by decapitation and the brain removed rapidly and kept on ice. The decapitation procedure was conducted without the use of anesthetic agents given the evidence that anesthesia may interfere with the AChE activity (Gomez et al., 2000; Pastuszko, 1980; Silva et al., 2005; Valadão et al., 2013; van Rijn et al., 2011). In order to expose the brainstem, the cerebellum was gently removed with tweezers through the disconnection of the cerebellar peduncles. After, two cross sections were performed, one at the level of the first cervical region and other at the end of the pons. The isolated brainstem was homogenized in ice, using a Potter-Elvehjem homogenizer (Tecnal, SP-Brazil), over 5 minutes in phosphate buffer (pH= 0.8, 0.1M, Dinâmica, SP-Brazil) and Triton X-100 (1% v/v, Neon, SP-Brazil) in a proportion of 20 mg of tissue for 1 mL of buffer. Thereafter, the homogenates were centrifuged at 7800G for 5 minutes at 4°C (Centrifuge 5804R – Eppendorf AG. Hamburg, Germany) and stored at -20°C for no longer than 48 hours until AChE assay was processed (see AChE.activity for description).

Chemoreflex activation

Chemoreflex activation was achieved by randomly assigned intravenous injections of potassium cyanide (KCN; 10, 20, 40, 80 µg/rat; Impex, SP, Brazil) according to Franchini & Krieger (1993). The peak hypertensive and bradycardic response was taken after each dose of cyanide.

Bezold-Jarisch reflex (BJR) activation

The BJR was activated by randomly assigned intravenous injections of phenylbiguanide (PBG, 1.5, 3.0, 6.0, 12 and 24 µg/kg, Sigma-Aldrich Brasil Ltda, RJ-Brazil) as described previously (Maretto et al., 2012; Uggere et al., 2000). The peak hypotensive and bradycardic response was quantified after each dose.

Baroreflex activation

According to the procedure described by Frigero et al. (2000) and Resstel et al. (2004), intravenous infusions of phenylephrine (50 µg/ml, Sigma Chemical) or sodium nitroprusside (100 µg/ ml, Commercial formulation, Nitrop, Hypofarma, MG-Brazil) were used to elicit baroreflex bradycardic and tachycardic responses,

respectively. The infusion pump (EFF 311, Insight, SP, Brazil) was adjusted for a flow of approximately 0.1 ml/min for phenylephrine and 0.2 ml/min for sodium nitroprusside infusions. Infusions did not exceed a volume of 0.1 ml over period of 30s and produced maximal pressor or depressor responses in the range of $\pm 40 - 50$ mmHg.

Evaluation of Baroreflex responses

Corresponding reflex responses in HR were measured at intervals of ± 5 mmHg over $\pm 40 - 50$ mmHg; these data were used to construct sigmoidal fitting curves (Head and McCarty, 1987; Sampaio et al., 1999) or linear regression curves as described previously (Resstel et al., 2004). The parameters obtained from the sigmoidal curves were: the tachycardia *plateau* (TP, bpm), corresponding to the maximum increase in HR due to the decrease in arterial pressure after nitroprusside injections; the bradycardia *plateau* (BP, bpm), corresponding to the maximum decrease in HR due to phenylephrine induced increase in pressure; the range of the reflex (bpm), representing the difference between the TP and the BP; the arterial pressure in the midpoint of the curve (AP₅₀, mmHg) and the average gain of the reflex (G, bpm/mmHg), given by the slope of the curve, which represented the sensitivity of the reflex. From the linear regression curves we extracted: the gain of the bradycardic and tachycardic curves, represented by the slope of the curves as well as the x-intercept of the regression lines.

Butyrylcholinesterase (BChE) activity

BChE activity was measured from the plasma samples taken before and after CPF (n=20) and saline (n=16) treatment. Five to seven samples were randomly selected from saline and CPF treated animals submitted to each reflex testing (see Experimental Protocol for description). The measurements were made with a commercial kit (K094, Quibasa, MG, Brazil), according to the colorimetric method described by Dietz et al. (1973), with modifications. These measurements were made in a semi-automated biochemical analyzer (TP Analyzer Plus, Thermoplate, China). The BChE activity was expressed in International Units (I.U./mL), where

1.0 I.U. of cholinesterase is equivalent to the amount of enzyme that hydrolyzes one mmol of substrate/minute/mL of serum at 37 °C.

Acetylcholinesterase (AChE) activity

Measurements of AChE were processed from brainstem samples of CPF and saline treated rats (n= 4 per treatment). The enzyme activity was determined using the original method described by Ellman et al. (1961) and modified by Lassiter et al. (2003). A volume of 135 µl of the brainstem homogenate was added to a cuvette containing the following reagents: 35 µl of 0.01M dithio-bisnitrobenzoic acid (DTNB), 10µl of 75mM acetylthiocholine (ATCh) and 820 µL phosphate buffer, pH 8.0, to make a final volume of 1 ml. The development of colour was analyzed at 412 nm, using a spectrophotometer (Evolution 300 – Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA). The acetylcholinesterase activity was expressed in milimoles of ATCh hydrolyzed per hour per milligram of protein. The protein content of brainstem homogenates was quantified by the method of Bradford (1976), using bovine serum albumin as standard.

Experimental Protocol

First, rats underwent the catheterization procedure (see above). On the next day, blood samples were collect and animals were then treated with saline or CPF and observed for up to 3 hours to monitor acute cholinergic signs of poisoning.

Recordings of baseline levels of MAP and HR as well as activation of the three neural reflexes were performed 24 hours after either chlorpyrifos or saline treatment. To avoid interference of multiple injections for reflex testing, 10 saline and 10 chlorpyrifos treated animals were independently tested for each reflex (Total = 30 animals/treatment). For all reflexes tested, a minimal interval of 10 minutes or complete recovery of basal values was observed between each dose assigned. Total recording time, including baseline recordings and reflexes testing, ranged from an hour to an hour and a half. At the end of reflex testing, a second blood sample was collected from each animal and four animals from each group were decapitated for brainstem collection. Experimental duration from dosing to sacrifice elapsed within 25 to 26 hours.

Statistical analyzes

The data were analyzed using GraphPad Prism software (version 5.0, USA) or Statistical Package for the Social Sciences version 20.0 for Windows (SPSS Statistics, Chicago, IL, USA). Acute toxicity signs were analyzed by Pearson's Chi-Square test. A two-way ANOVA for repeated measures (RM) followed by Bonferroni *post hoc* test was used for analysis of changes in MAP and HR after chemoreflex and BJR activation as well as for the statistical analysis of the BChE activity. The baroreflex parameters and the AChE activity within the brainstem were analyzed by the Student's *t* test. The level of significance was set at * $P < 0.05$. The data were expressed as mean \pm standard error of the mean (SEM).

Results

Acute toxicity

The percentage of animals that presented acute toxicity signs as well as the latency time for their appearance is shown in Table 1. The animals treated with CPF presented ataxia ($X^2= 43.07$), tremor ($X^2= 62.22$), lacrimation ($X^2= 10.141$), salivation ($X^2= 72.381$), tetany ($X^2= 11.429$) and a high frequency of urination and defecation ($X^2= 40.833$). The only signs observed in saline treated animals were urination and defecation. The signs with lowest latency time in CPF treated animals were urination and defecation (Table 1). No visual signs of toxicosis were present 24 hours after CPF dosing.

Effects of CPF treatment on baseline values

No difference in baseline values of MAP (CPF: 117 ± 1.7 ; Saline: 118 ± 1.7 , $t=0.507$; $P> 0.05$) or HR (CPF: 322 ± 6.7 ; Saline: 339 ± 5.8 , $t=1.933$; $P>0.05$) were observed between CPF and saline treated animals 24 hours after dosing.

Chemoreflex responses

Figure 1 (A) shows a typical tracing of the KCN induced responses in CPF and saline treated animals. The hypertensive and bradycardic responses evoked by chemoreflex activation were significantly reduced in CPF treated animals.

The grouped data of the chemoreflex responses is presented in Figure 1 (B). The analysis of the changes in MAP induced by the KCN injections in CPF and saline-treated animals showed an effect for the treatment ($F_{(1,18)}= 26.62$; $p<0.0001$), for the KCN doses ($F_{(3,18)}= 151.7$; $p<0.0001$) and also showed interaction between the treatment and the doses ($F_{(3,18)}= 10.45$, $P<0.0001$). The *post hoc* analysis showed that the hypertensive response of CPF treated animals induced by chemoreflex activation was decreased for the doses of $20\mu\text{g}$ ($P<0.001$), $40\mu\text{g}$ ($P<0.05$) and $80\mu\text{g}$ ($P<0.001$) when compared with the same doses in saline-treated rats (Figure 1B, left panel).

Concerning the HR changes induced by KCN, the analysis showed a difference for the treatment ($F_{(1,18)} = 11.40$, $P = 0.0034$) and for the different doses of KCN ($F_{(3,18)} = 150.50$, $P < 0.0001$) as well as an interaction between the treatment and the KCN doses ($F_{(3,18)} = 5.60$, $P = 0.002$). The *post hoc* analysis showed that the chemoreflex bradycardic response in CPF treated animals was decreased at doses of 20 μ g ($P < 0.001$), 40 μ g ($P < 0.05$) and 80 μ g ($P < 0.005$) when compared with the same doses in saline-treated rats.

Cardiovascular responses induced by the Bezold-Jarisch reflex (BJR) activation

As with the chemoreflex, the BJR responses were also decreased in the CPF treated rats (Figure 2A). The grouped data are presented in Figure 2B. Concerning the MAP changes induced by BJR activation, a difference was observed for treatment ($F_{(1,18)} = 16.47$; $p < 0.0007$) and for the doses of PBG ($F_{(4,18)} = 103.9$; $p < 0.0001$) but there was no interaction between the treatment and the PBG doses ($F_{(4,18)} = 0.73$, $P < 0.57$). The *post hoc* analysis showed an impairment of the hypotensive response of BJR in CPF treated animals at the doses of 1.5 μ g ($P < 0.001$) and 3 μ g ($P < 0.05$) (Figure 2B, left panel).

Additionally, our HR analysis showed a difference for the treatment ($F_{(1,18)} = 18.64$, $P = 0.0004$), PBG doses ($F_{(4,18)} = 289.0$, $P < 0.0001$) and an interaction between the treatment and the different doses employed to activate the BJR ($F_{(4,18)} = 3.29$, $P = 0.0155$). The *post hoc* analysis showed that the bradycardic response induced by BJR activation was decreased in CPF treated rats at the doses of 1.5 μ g ($P < 0.001$), 3 μ g ($P < 0.05$), 12 μ g ($P < 0.05$) and 24 μ g ($P < 0.001$) (Figure 2B, right panel).

Baroreflex responses

A typical baroreflex responses of CPF and saline treated animals is shown in Figure 3A. The reflex bradycardic response of CPF treated animals was reduced in magnitude when compared with saline treated animals.

The pooled baroreflex sigmoidal data are presented in figure 3B. There was a reduction in the bradycardia *plateau* (CPF: -34.0 ± 9.13 ; Saline: -112.3 ± 14.67 ; $t = 4.527$; $P < 0.01$), the range (CPF: 96.6 ± 11.76 ; Saline: 183.9 ± 25.54 ; $t = -3.105$, $P < 0.01$) and the gain (CPF: -0.61 ± 0.12 ; Saline: -2.37 ± 0.42 ; $t = 3.978$; $P < 0.01$) of the baroreflex of CPF treated animals when compared with saline treated rats (Figure 3B, left panel). The linear regression curves showed that the gain of bradycardic response was significantly reduced in CPF treated animals compared to control (CPF: -1.3 ± 0.23 ; Saline: -2.9 ± 0.43 ; $t = 3.277$; $P < 0.01$) (Figure 3B, right panel). In contrast, both the AP_{50} (CPF: -12.7 ± 3.37 ; Saline: -1.8 ± 4.71 ; $t = -1.881$; $P > 0.05$) and the tachycardia plateau ($t = -0.863$; $P > 0.05$) were not different between groups (Figure 3B, left panel). No differences were observed in the x-intercept of the bradycardic curve (CPF $30 = 7.35 \pm 1.34$; Saline = -3.04 ± 5.35 ; $t = 1.886$; $P > 0.05$) or in the gain and x-intercept of the tachycardic curve (Gain: CPF = -1.99 ± 0.28 ; Saline = -2.54 ± 0.41 ; $t = 1.098$, $P > 0.05$; x-intercept: CPF = -7.10 ± 2.06 ; Saline = -4.66 ± 1.37 , $t = -0.984$; $P > 0.05$).

Butyrylcholinesterase activity

BChE activity after CPF treatment was different relative to control ($F_{(1,34)} = 26.89$, $P = 0.0001$), as well as for the time ($F_{(1,34)} = 85.20$, $P = 0.0001$) and showed an interaction between treatment and time ($F_{(1,34)} = 56.77$, $P = 0.0001$). The *post hoc* analysis showed a reduction in the BChE activity of CPF treated animals in comparison with the pre-treatment condition (BChE_{before} = 210.8 ± 15.40 ; BChE_{after} = 46.7 ± 7.85 ; $P < 0.0001$) (Figure 4).

Acetylcholinesterase (AChE) activity

The AChE activity within the brainstem of CPF treated animals was reduced when compared with saline treated rats (Saline = 2.1 ± 0.15 ; CPF = 0.7 ± 0.06 $\mu\text{mol/h/mg}$ of protein; $t = 8.725$, $P < 0.0001$) (Figure 5).

Discussion:

In the present report, we showed that acute intoxication with chlorpyrifos (CPF) impairs the cardiovascular responses of three distinct neural reflexes tested. We showed that a sub-lethal dose of CPF induced notorious signs of acute toxicity observed during the clinical evaluation of the animals after poisoning. These effects were also accompanied by a marked inhibition of both plasma BuChE activity and AChE within the brainstem.

For the intoxication procedure, we used a commercial formulation of CPF and choose the maximum tolerated dose that did not produce lethality. The use of a commercial formulation of CPF for animal's treatment, instead of the pure compound, was adopted to provide similar conditions to the clinical setting. Although, it could be argued that interference played by other ingredients present in the formulation may bring some uncertainty to the data, the acute toxicity signs observed in CPF treated rats as well as the inhibition of the AChE and BChE activities after CPF dosing seems to support the effects to be mainly attributed to the OP compound. The choice of a high, although sub-lethal dose of CPF, was to simulate clinical conditions of a massive acute intoxication by OP compounds, which usually occurs among farmers, frequently as a result of self-poisoning in suicides attempts (Bochner, 2007; Jeyaratnam, 1990; London et al., 2005). The effectiveness of simulating this condition could be ascertained by the frequency of acute toxicity signs presented in the CPF treated animals. It is usual in the experimental trials involving studies with OP's exposure to evaluate the acute toxicity signs exerted by these compounds as an index of the treatment effectiveness. These signs of toxicity with CPF are similar to those reported previously (Howard et al., 2007; Moser et al., 1998; Nostrandt et al., 1997; Ruiz-Muñoz et al., 2011).

The effectiveness of poisoning was also confirmed by the reduction in plasma BuChE activity, when compared to the pre-poisoning condition and with control animals. The assay of the BuChE cholinesterase activity is a proven sensitive

method for monitoring exposure to OP compounds (Do Nascimento et al., 2017; Eddleston et al., 2008; Maretto et al., 2012a; Oliveira-Silva et al., 2000; Pohanka, 2013). Interestingly, the measurement of BuChE has been proposed as a potential marker for predicting cardiovascular risk (Sulzgruber et al., 2015; Sun et al., 2016), which further reinforces the importance of measuring this enzyme activity in studies involving OP exposure and cardiovascular effects. Measurement of the AChE activity within the brainstem, where the main nuclei mediating cardiovascular reflexes are located (for review Dampney, 2016; Salman, 2016), complemented the results obtained for the plasma BuChE. Animals treated with CPF presented a marked inhibition of brainstem AChE activity when compared with control animals. Nostrandt and colleagues (1997) showed that rats acutely intoxicated with CPF, using a similar dose to that of our study, presented a 60% inhibition of the AChE activity in samples of the pons/medulla 24 hours after the intoxication procedure; such reduced levels are very similar to that observed in the present study. Therefore, inhibition of brainstem AChE confirmed the effectiveness of the treatment which correlates with the effects observed on cardiovascular reflex function.

The acute exposure to CPF impaired the bradycardic response of all reflexes tested. Previous work from our group showed that acute sub-lethal exposure to methamidophos, another OP compound, also impaired the bradycardic response of the chemoreflex and of the BJR in rats (Maretto et al., 2012). Considering that the reflex-induced reduction in HR is mostly dependent on vagal activation (Aviado and Guevara Aviado, 2001; Chianca and Machado, 1996; Costa et al., 2014; Dampney, 2016; Gourine et al., 2016; Head and McCarty, 1987; Machado, 2001; Salman, 2016; Simms et al., 2007; Spyer and Gourine, 2009) these results indicate that exposure to CPF impaired the parasympathetic component of these reflex responses. Multiple mechanisms could be involved: First, inhibition of AChE, with consequent cholinergic overstimulation might be inducing cholinergic receptors desensitization, once it is established that sustained agonist cholinergic stimulation produces desensitization of both nicotinic and muscarinic receptors (Baumgold et al., 1989; Giniatullin et al., 2005; Richelson, 1978). It is well known that either acute

or repeated exposure to OPs induces resistance to cholinergic agonist induced effects (Costa et al., 1982). Second, the effect observed may not be exclusively related to inhibition of acetylcholinesterase, as OP compounds can directly bind to nicotinic and muscarinic receptors (Huff et al., 2001; Katz et al., 1997; Pope, 1999; Silveira et al., 1990; Udarbe Zamora et al., 2008). Particularly, it has been shown that the CPF active metabolite, chlorpyrifos oxon (CPO) binds to M₂ cardiac muscarinic receptor through diethylphosphorylation, which affects agonist binding to the receptor (Bomser and Casida, 2001; Howard et al., 2007; Howard and Pope, 2002). Therefore, the decrease in the bradycardic response of the three reflexes tested might be due to direct inactivation of cardiac muscarinic receptors by CPO.

It cannot be excluded, however, that exposure to CPF might also affect sympathetic function. Although in the linear regression curves only the bradycardic gain was significantly reduced in CPF treated animals, the sigmoidal fitting analysis of the baroreflex function CPF poisoned animals showed a reduction of the baroreflex gain as well as of the reflex range. While the baroreflex range is made up of approximately 60% contribution from vagus compared with 40% of the sympathetic, the gain is slightly more dependent on the sympathetic component than the parasympathetic (Head and McCarty, 1987). Accordingly, the changes observed in baroreflex function might suggest interference with sympathetic function. This interference could involve modulation of sympathetic ganglia, where ganglionic neurotransmission is mainly conducted through the cholinergic system (Vernino et al., 2008) or involve modulation within the central nervous system. In this regard, evidence has been provided showing the role of central cholinergic neurons in the regulation of the sympathetic tone (Buccafusco, 1996). Additionally, exposure to soman, another OP compound, induced a short lasting pressor response mediated by an increase in sympathetic activity due to central muscarinic receptors activation (Brezenoff et al., 1984). Therefore, it is possible that the CPF exposure either through desensitization or directly binding to cholinergic receptors within brainstem nuclei or at the ganglia might be dampening sympathetic function. This hypothesis is reinforced by the CPF-induced reduction in the chemoreflex hypertensive response, which is dependent on sympathetic activation to vessels

(Costa et al., 2014; de Burgh Daly, 1985; Guyenet, 2006; Spyer and Gourine, 2009). In contrast to our data, Maretto and colleagues (2012) found no changes in the chemoreflex induced hypertensive response after exposure to methamidophos. However, these differences may relate to the effects played by different OP compounds on vascular resistance. Experimental results from Guvenc Tuna et al. (2011) have shown that exposure to CPF but not to dichlorvos, another OP compound, impairs elastic fibers of the aorta. In fact, differential targets other than the common inhibition of AChE has been shown to produce differential toxicities among different OP compounds (Chaudhuri et al., 1993; Pope, 1999). The reduction in the hypotensive response of the BJR of CPF treated animals, may be secondary to the reduction in the bradycardic response after CPF poisoning. In fact, Chianca and Machado (1996) showed that methylatropine administration to rats not only blocked the BJR bradycardic response but also abolished the reflex-induced hypotension, suggesting that this response is a consequence of the reflex induced reduction in HR and cardiac output.

Contrary to the modulation in the reflex responses, baseline levels of MAP and HR were not significantly different between CPF and control animals, although there was a tendency for a reduction in basal heart rate of CPF poisoned animals (CPF: 322 ± 6.7 vs Saline: 339 ± 5.8). Different effects on arterial pressure and heart rate have been observed in clinical reports and in experimental studies with OPs. Hypotension, hypertension, bradycardia and tachycardia have all been reported in patients acutely intoxicated with OP compounds (Anand et al., 2009; Davies et al., 2008; Saadeh et al., 1997). However, as these effects involve exposure to different OP compounds, at different doses, it is hard to compare the clinical data with the present results. A great variation in the experimental data, which may be related to differences in the OP compound, dose, route of administration, exposure duration, species, methods for monitoring parameters and presence/absence of anaesthesia. Intravenous administration of paraoxon as well as of soman to Sprague-Dawley conscious rats produced an increase in blood pressure that lasted up to 8 hours, which was associated with a light bradycardia (Bataillard et al., 1990). Brezenoff and colleagues (1984) showed that administration of soman to

anaesthetized Sprague-Dawley rats produced a hypertensive response that lasted for about 90 minutes, with either bradycardia or tachycardia depending on the route of administration. On the other hand, intravenous administration of a lethal dose of diazinon or fenthion to anaesthetized rats induced bradycardia followed by a decline of blood pressure (Kojima et al., 1992). Subcutaneous administration of a sub-lethal dose of CPF to Long-Evans rats lead to an elevation in heart rate which lasted for approximately 72 hours (Gordon, 1994). Results from the same research group employing oral administration of lower doses of CPF to the same species induced sustained hypertension that lasted approximately 32 hours and a short lasting bradycardia (Gordon and Padnos, 2000). Anthon and Campanã-Salcido (2011), also found a hypertensive response, but with an increase in heart rate that lasted up to 24 hours in Wistar rats acutely exposed to CPF for three days. Additionally, data from Smith and Gordon (2005) comparing CPF exposure in SHR and Wistar Kyoto (WKY) rats, showed much more pronounced and long lasting hypertensive and tachycardic effects in SHR than in WKY animals. The authors discuss that more pronounced effects observed in SHR rats might relate to a major role played by the central cholinergic modulation in the hypertensive state. Although, it was not the aim of our study to monitor the immediate changes in MAP and HR after exposure it seems that if any changes in baseline values happened after exposure they occur within the first hours after exposure. Additionally, the difference among the species, OP compounds, the comparison with pre-treatment condition, the differences in duration, route and dose of exposure might account for the differences observed as well as modulation of an array of reflexes with opposing actions.

Many authors have attempted to establish mortality predictors for acute OPs poisoning. It is consensus that even among patients receiving intensive care, such as ventilatory support, gastric lavage and standard therapy with the antidotes, atropine and pralidoxime, mortality is still high for these groups of individuals. The mortality rate in intensive care units is around 10-50% even with mechanical ventilation (Banday et al., 2015; Munidasa et al., 2004; Tang et al., 2016). Munidasa et al (2004) reported that the mortality rate among the patients studied

was 50%, even with mechanical ventilation and oxygen supplementation. The authors suggested that the primary contributor to the patient's mortality when respiratory failure is controlled by mechanical ventilation, would be an overall impairment of myocardial function together with direct cardiac effects of the OPs. This is consistent with our finding that two of the reflexes studied (chemo- and BJR) are both protective to the heart. Reducing the potency of protective reflexes may well increase myocardial vulnerability to OPs. Indeed, cardiac injury is an important cause of death in acute OP poisoning (Aghabiklooei et al., 2013; Anand et al., 2009). In addition, an impairment of the chemoreflex function may contribute to worsening the respiratory state after poisoning. Increase in pulmonary secretions after OP poisoning impairs gas exchange in the lungs (King and Aaron, 2015; Marrs, 1993). In fact, Gaspari and Paydarfar (2012, 2007) reported that mechanical ventilation was not capable of preventing the mortality of rats given a lethal dose of dichlorvos. According to the authors, even mechanical ventilation with normoxic supplementation was inefficient in maintaining the oxygen blood saturation, leading the animals to a hypoxic state. Additionally, with the worsening of the animal's respiratory function, no changes in the baseline cardiovascular parameters (blood pressure and heart rate) were observed, which strongly suggests that the chemoreflex responses are presumably impaired in OP poisoning.

Important clinical implications have been attribute to acute OP poisoning not only during the acute event but also in long term. Hung and colleagues (2015) showed in a 12 years' retrospective cohort study involving patients' victims of acute OPs poisoning in Taiwan, that acute OP poisoning is a significant risk factor for development of long term cardiovascular diseases such as arrhythmia, coronary artery disease (CAD) or congestive heart failure (CHF). The mechanisms involved in the long-term effects are not well understood, but the impairment observed in our study could add information on that matter once it is well known that impairment in these neural reflexes are important factors associated with the development or maintenance of pathological states such as hypertension, obstructive sleep apnea, coronary heart disease, congestive heart failure and

stroke (Fernandez et al., 2015; Guyenet, 2006; Kara et al., 2003; Meyrelles et al., 1997; Molkov et al., 2014; Persson, 2005; Simms et al., 2007; Vasquez et al., 1997) However, whether the impairment in the reflexes function observed in the present study is long lasting, is a subject for further investigations.

Conclusions:

Our results showed that acute sub-lethal exposure to CPF impairs the cardiovascular responses of three regulatory and protective neural reflexes tested. These effects are associated with a marked inhibition of the plasma BuChE and of the AChE within the brainstem. The results observed may have important short and long term clinical implications in OP poisoning, once if death does not happen as a consequence of cardiorespiratory complications, survival after poisoning may condition the body to be more vulnerable to cardiovascular risk.

Conflict of interest statement:

The authors declare that there is no conflict of interest.

Financial Support:

This research was funded by FAPES foundation (Grant number: 67650856/15) and by FAPES foundation in cooperation with Research Councils UK – CONFAP (Grant number: 68911050/2015). ISAF was recipient of CAPES fellowship.

Acknowledgments:

Brainstem AChE assays were performed at the Laboratório Multiusuário de Análises Moleculares (LABIOM) of the Health Sciences Center of the Federal University of Espírito Santo.

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Figure 1: A. Representative recordings showing changes induced by intravenous injection of KCN doses (10, 20, 40 and 80 µg/rat) in pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) in CPF and saline treated animals. **B.** Grouped data showing changes (Δ) in MAP (left) and HR (right) induced by administration of KCN doses (10, 20, 40 and 80 µg/rat) in CPF (n=10) and saline (N=10) treated rats *P<0.05 and **P<0.01 indicates statistical difference between saline and CPF for each respective dose. Two-way ANOVA for repeated measures, Bonferroni's *post hoc* test.

Figure 2. A. Representative recording showing changes induced by intravenous injection of phenylbiguanide (PBG) doses (1.5; 3; 6; 12 and 24 µg/kg) in pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) in CPF and saline treated animals. **B.** Grouped data showing changes (Δ) in MAP (left) and HR (right) induced by administration of PBG doses (1.5; 3; 6; 12 and 24 µg/kg) in CPF (n=10) and saline (N=10) treated rats *P<0.05 and **P<0.01 indicates statistical difference between saline and CPF for each respective dose. Two-way ANOVA for repeated measures, Bonferroni's *post hoc* test.

Figure 3: A. Representative recording showing changes induced by intravenous infusion of phenylephrine or sodium nitroprusside in pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) in CPF and saline treated animals. **B. Left panel:** Sigmoid curves correlating changes (Δ) in mean arterial pressure (MAP, mmHg) with changes (Δ) in heart rate (HR, bpm) in animals treated with saline (continuous line, n=10) or chlorpyrifos (CPF 30 mg/kg, dashed line, n=10). **Right panel:** Linear regression curves showing the baroreflex sensitivity for the bradycardia and tachycardia responses of animals treated with saline (continuous line, n=10) or chlorpyrifos (CPF 30 mg/kg, dashed line, n=10). Student's *t* test.

Figure 4. Butyrylcholinesterase activity (UI/mL) of rats before and after treatment with saline (n=16) or chlorpyrifos (CPF, 30 mg/kg, n=20). **P<0.01 before and after CPF treatment. Two-way ANOVA for repeated measures, Bonferroni's *post hoc* test.

Figure 5. Acetylcholinesterase (AChE) activity (umol/h/mg of protein) within the brainstem of rats treated with saline (n= 4) or chlorpyrifos (CPF, 30 mg/kg, n= 4). *t* = 8.725, ** P<0.01 saline vs CPF. Student's *t* test.

Table 1. Manifestation frequency, in percentage, and latency time, in minutes, for the acute toxicity signs after treatment with CPF (30 mg/Kg; n=40) or saline (0.9%; n=40).